EBOV protection is supported by T cell-dependent humoral responses but is not requisite for survival.

Christopher L. Cooper, Karen A. Martins, Sabrina Stronsky, David Langan, Jesse Steffens, Sean Van Tongeren, and Sina Bavari

Abstract

Despite agreement across Ebola virus (EBOV) vaccine platforms of a requisite role for antibody-dependent protection and extensive efforts in development of antibody therapy against lethal EBOV infection, the establishment of protective EBOV B cell responses has yet to be fully described. Previously, we demonstrated that vaccination of mice, guinea pigs, or nonhuman primates with virus-like particles (eVLP) expressing EBOV glycoprotein (GP) elicited full protection. More recently, we illustrated that inclusion of the Toll-like receptor 3 agonist and clinical grade adjuvant, Hiltonol, enhanced GP-specific antibody titers and durable EBOV protection. Here in, we outline that the cellular events mediating EBOV humoral responses are exclusively generated from T-cell dependent mechanisms. We show that Hiltonol both augmented and sustained eVLP-mediated GC B cell formation and increased antigen-specific B cell frequencies. In addition, we define the *in-vivo* requirement of T-cell follicular help and CD40-CD40L signaling in establishing EBOV GP antibody responses. Finally and unexpectedly, we demonstrate that while EBOV antibody responses can enhance protection, survival in the complete absence of antibodies can be achieved; however, B cells are obligate.

Introduction

Humoral immune responses are a hallmark for successful clearance of pathogens and protective vaccines. Antibody-mediated control (i.e. opsonization, neutralization, antibody-dependent cell-mediated cytotoxicity) of infectious diseases, such as Clostridium, HIV, Influenza, and RSV, has been

well-documented. The generation of high-affinity class-switched antibodies is dependent upon several key initiating events including: 1) the onset and duration of germinal center (GC) B cell reactions 2) activation-induced cytidine deaminase (Aicda; AID) mediated somatic hypermutation and isotype class-switching, and 3) T cell follicular help in the selection and differentiation of GC B cells to form the antigen (Ag)-specific B cell compartments (i.e. plasma cells, memory). The culmination of these actions, as well as others, can directly shape the quality and quantity of the humoral response.

While considerable efforts using multiple antigen systems have provided a framework for understanding the establishment of humoral immunity, the translation of these events to complex viral antigens or emerging pathogens have been limited. The recent 2014 Western Africa Ebola virus (EBOV) epidemic, which affected nearly 28,000 individuals and claimed a reported 12,000 lives within a year, has catalyzed the advancement of several Ebola virus medical countermeasures, some of which had been in development for decades. Multiple FDA vaccine clinical trials centered on the EBOV trimeric glycoprotein (GP) have initiated, including Phase III trials with the replicating VSV-G GP Merck vaccine. However, several fundamental gaps exist in our understanding of how EBOV protective responses are established and a definitive immune correlate has yet to be defined.

Founded on non-human primate and rodent studies, as well as on the preclinical success of therapeutic antibodies (ZMapp, ZMab) in treatment of EBOV infection, humoral immunity is thought to be critical for protection against EBOV infection. However, *in-vivo* studies to define how B cell immunity is established and the direct requirement of these responses in EBOV protection have not been fully addressed. In this study, we utilized viral-like particles, which offer a natural EBOV mimicry (eVLP) and provide protective vaccine immunity, to interrogate the establishment of GP-specific humoral immunity. We define the cellular events required to establish EBOV-GP antibody responses. Further, we provide insight into how adjuvants promote protective humoral immune responses. Finally and unexpectedly, we found that

vaccine induced EBOV protection can occur in the complete absence of antibodies. Collectively, these findings offer increased granularity for establishment of EBOV B cell responses and provide additional avenues to approach rationale EBOV vaccine design.

Hiltonol promotes continual and sustained EBOV antibody responses

Ebola viral-like particles offer structurally identical, non-infectious surrogate virus systems which have been essential in defining EBOV host-pathogen interactions. In addition, eVLP have been shown to offer complete EBOV protection across multiple species when utilized as a vaccine. Using the murine model, we have recently demonstrated that eVLP-mediated EBOV protection is associated with a robust Th1-like IgG class switched antibody response. Further, we illustrated that both protection and antibody titers wane several months following prime-boost vaccination; however, inclusion of the clinical grade dsRNA (pIC:LC) adjuvant, Hiltonol, enhanced humoral responses and resulted in durable protection.

To further extend our understanding of eVLP humoral immunity and the impact of adjuvant on these responses, we examined the antibody kinetics associated with the acute phase of EBOV protection, defined as protection conferred 28 days post final vaccination (d.p.v.). Animals were administered eVLP (intramuscular, im) with or without Hiltonol using a prime-boost schedule at three week intervals (Day 0, Day 21), and EBOV GP-specific antibody responses were determined during the vaccination course. As previously described, eVLP vaccination resulted in a rapid IgG antibody response with an approximate log-fold increase in titers with inclusion of adjuvant (Fig 1a). However, we observed that during acute phase protection, eVLP antibody responses appeared to plateau following vaccination, while inclusion of Hiltonol resulted in a continual increase of total IgG titers up to the time of acute (D49) EBOV challenge (Fig 1a). Consistent with the rise in total EBOV GP-specific IgG titers, we observed Hiltonol enhanced EBOV neutralization during the course of vaccination (Fig 1b). While similar levels of EBOV neutralization (~50% reduction) were detectable as early as D14 following prime vaccination within both groups,

increased neutralization was apparent by D49 (28 d.p.v.) in the context of adjuvant (Fig 1b). As we have recently reported, durable IgG responses (> 5 months post vaccination) declined within eVLP animals while Hiltonol offered sustained humoral responses (Fig 1c, top). Of note, after a single, two or three vaccinations, EBOV GP-specific IgM responses were only marginally detected and required the presence of adjuvant (Fig 1c, bottom).

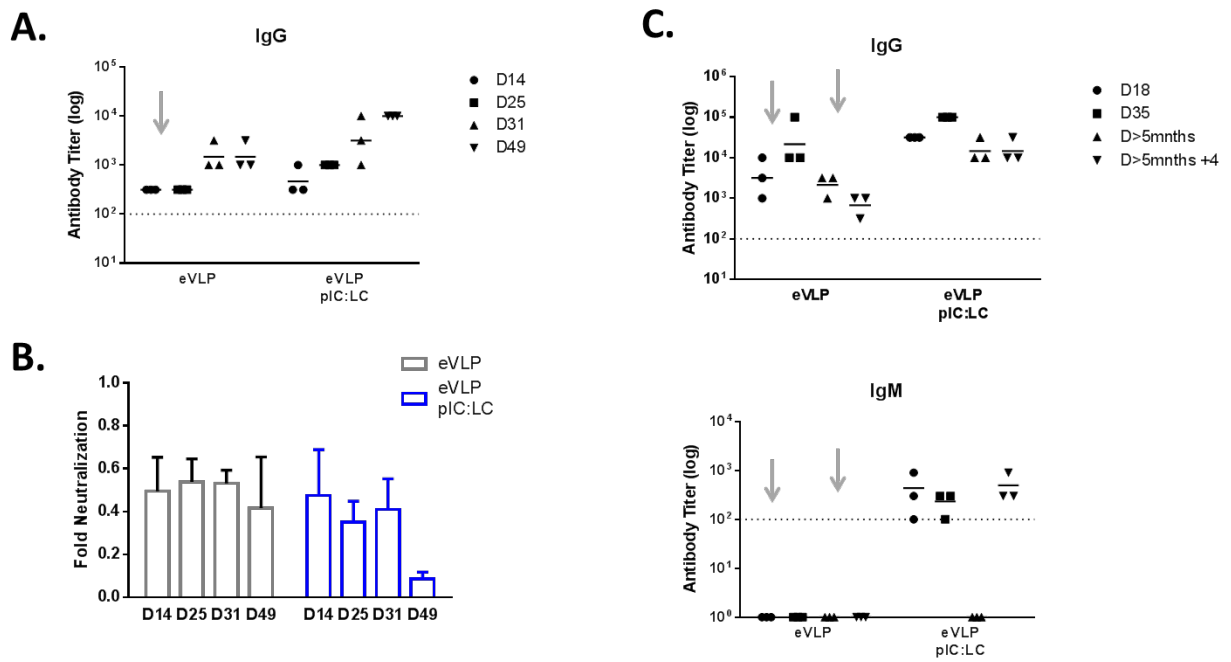

Figure 1. Characterization of short-term and memory EBOV GP antibody responses following eVLP vaccination. C57BL/6 mice were vaccinated with 10 ug (GP content) of VLP or 10 ug of VLP with 10 ug of Hiltonol at Day 0, D21 and D>5 months. Serum was collected at the indicated time-points and EBOV-GP specific IgM and IgG responses were measured by ELISA. A,B) EBOV-GP specific IgG titer (A) and viral neutralization (B) during acute phase protection. C) Durable IgM and IgG antibody responses. Titers were calculated by reciprocal end-point dilutions with background set at control absorbance + 0.2 O.D. (n=3). Arrows indicate vaccination time points.

eVLP-mediated germinal center formation.

Production of high affinity class-switched antibodies is dependent upon germinal center (GC) formation within the spleen and secondary lymphoid organs; however, the requirement of GC reactions in the generation of protective EBOV antibody responses is poorly defined. We therefore first determined if eVLP humoral responses were generated through GC reactions. Animals vaccinated (im) with eVLP

displayed an approximate three-fold increase in draining lymph node (dLN, popliteal) cellularity at D10 following prime-vaccination, while the inclusion of Hiltonol resulted in an approximate seven- fold increase (Fig 2a). This increase of cellularity associated with eVLP vaccination was detected as early as Day 3 (data not shown). With both eVLP and adjuvanted vaccinations, GC B cells (B220+GL7+CD95+) were clearly identified within the dLN; however, GC B cell frequencies were increased in the presence of Hiltonol (Fig 2b). We next determined the impact of vaccine boost on eVLP-mediated GC reactions. As with prime vaccination, GC B cells were detected within the dLN on Day 28 (D7 post-boost), and, consistent with prime vaccination, inclusion of adjuvant significantly augmented the relative frequency of GC B cells during the prime-boost schedule (Fig 2c, left panel). Additional phenotypic characterization of the B cell compartment supported the induction of activated class-switched (B220+GL7+CD95+CD38lowIgD- IgM-) B cells following eVLP vaccination with heightened frequencies observed by addition of Hiltonol (Fig 2c, right panel). Noteworthy and in line with a continued increase of EBOV GP-specific antibody titers seen in adjuvanted animals, the relative frequency of GC B cells appeared to increase following boosting with adjuvant while eVLP vaccinated animals displayed similar GC B cell frequencies following both prime and boost (Fig 2b,c). eVLP-mediated GC reactions were still present within the dLN at 4 weeks post-boost (D49) with a consistent increase in GC B cell frequencies observed in the presence of adjuvant (Fig. 2d). As productive GC reactions result in the generation of antibody secreting cells (ASC), we measured the frequency of antigen-specific B cells reactive against EBOV GP. eVLP GP-specific B cells were below our assay detection level after prime vaccination (data not shown); however, we observed an approximate five-fold increase in ASC frequencies with inclusion of Hiltonol following boosting (Fig 2e). These GP-specific B cells were still detectable up to D49 in the presence of adjuvant, but approached our lower limit of detection for eVLP vaccinations(data not shown). Collectively, we demonstrate eVLP-mediated protection is associated with GC formation and

that addition of the dsRNA adjuvant, Hiltonol, resulted in a consistent and sustained rise in GC B cell frequencies. Further, this phenotype extends to increased generation of EBOV GP-specific ASC.

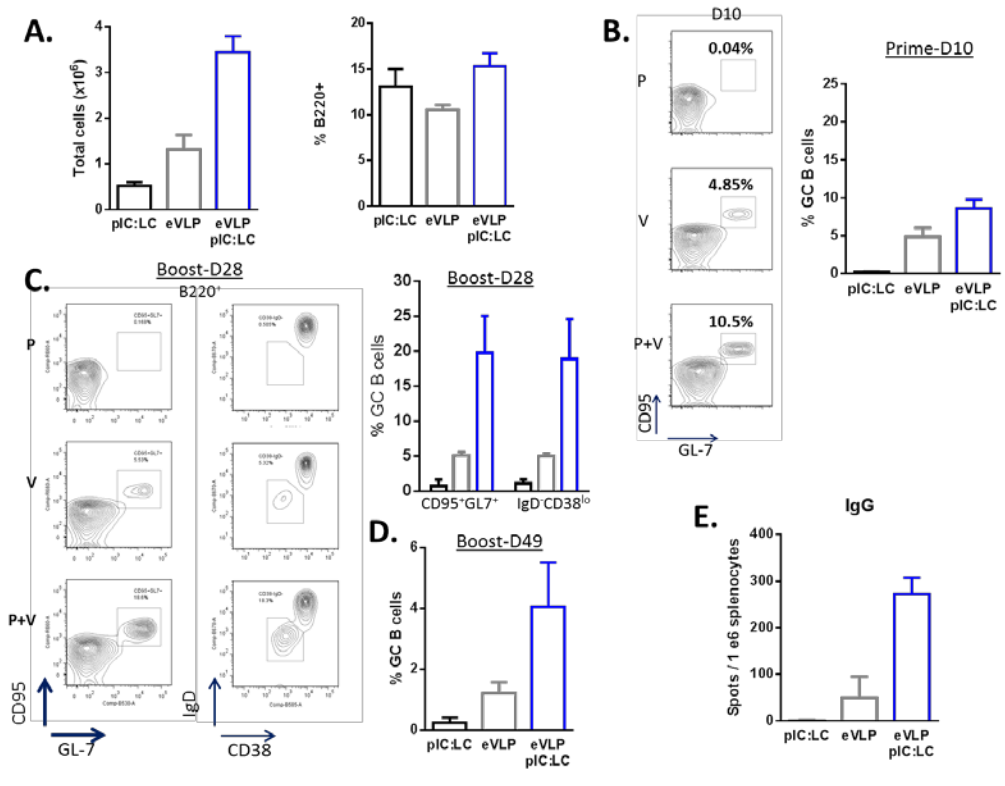

Figure 2. Hiltonol augments and sustains VLP-mediated Germinal Centers. C57BL/6 mice were vaccinated via I.M. with 10 ug (GP content) of eVLP or 10 ug of eVLP with 10 ug of PolyICLC and draining lymph nodes (DLN) were isolated. Single cell suspensions were then stained with B220, IgD, IgM, CD38, CD95, GL-7 and live/dead dye and collected on a BD FACS Canto II. A) left, D10 DLN cellularity right, relative percentage of B220+ population in D10 DLN. B) Representative FACS plot and relative percentage of D10 DLN B220+CD95+GL7+ GC B cells. D) Representative D28 (D7 post-boost) FACS plot and relative frequency of B220+CD95+GL7+ GC B cells (left panel and right top) and of B220+CD38loIgD-IgM- B cells (right panel and right top). E) Relative percentage of D49 (D28 post-boost) DLN B220+CD95+GL7+ GC B cells. E) Relative frequency of D25 (D4 post-boost) EBOV-GP specific B cells by ELISPOT in the spleen. Similar results were observed in multiple experiments with a minimum of 3-5 mice/group. (mean, s.e.m; n=3/5)

T cell-dependence on eVLP-mediated humoral immune responses

Several reports have demonstrated a requirement for both effector CD8 and CD4 T cell responses in protective EBOV vaccination. More recently, we have demonstrated that eVLP vaccination induces robust CD4 T cell effector functions and that CD8 T cells were dispensable for protection. While effector

T cell responses in protective EBOV immunity have been described, the role of T cells supporting humoral responses has yet to be fully defined. As the quality and quantity of GC B cell formation is dependent on T follicular help (T_{FH}), we determined the induction of this T cell compartment following eVLP vaccination. Consistent with the relationship between T_{FH} and the formation of GC reactions, eVLP-mediated T_{FH} (CD3+CD4+PD-1+CXCR5hi) cells were generated during vaccination (Fig. 3a,b). Moreover, the relative size of the T_{FH} compartment was increased with addition of adjuvant, suggesting Hiltonol augments humoral immunity through promoting T cell follicular help and GC B cell reactions (Fig. 3a,b). Consistent with cellular phenotyping, high levels of ICOS was detectable on T_{FH} cells with a subtle increase of expression observed with adjuvant (Fig 3c).

Given that CD40-CD40L interactions between GC B cells and T_{FH} cells, respectively, are required to induce productive T cell dependent (TD) antibody responses, we determined the direct *in-vivo* contribution of this pathway during EBOV vaccination. To address this question, we used the TNF superfamily receptor 5 (CD40 -/-) knockout mouse model which have defective TD humoral immune responses while maintaining capabilities to mount T cell independent (TI) antibody responses. WT and CD40 -/- animals were prime-boost vaccinated (D0, D21) with eVLP and EBOV GP-specific antibody titers were determined on Day 35. Consistent with previous results, WT animals vaccinated with eVLP displayed a robust IgG titer; however, CD40 -/- animals displayed a complete absence of GP-specific IgG antibody responses (Fig. 3d, bottom). Further, Hiltonol induction of eVLP IgM responses was also impaired in the absence of T cell help (Fig. 3d, top). Together, these findings support a requisite role for T cell dependent mechanisms in the generation of eVLP-mediated antibody responses.

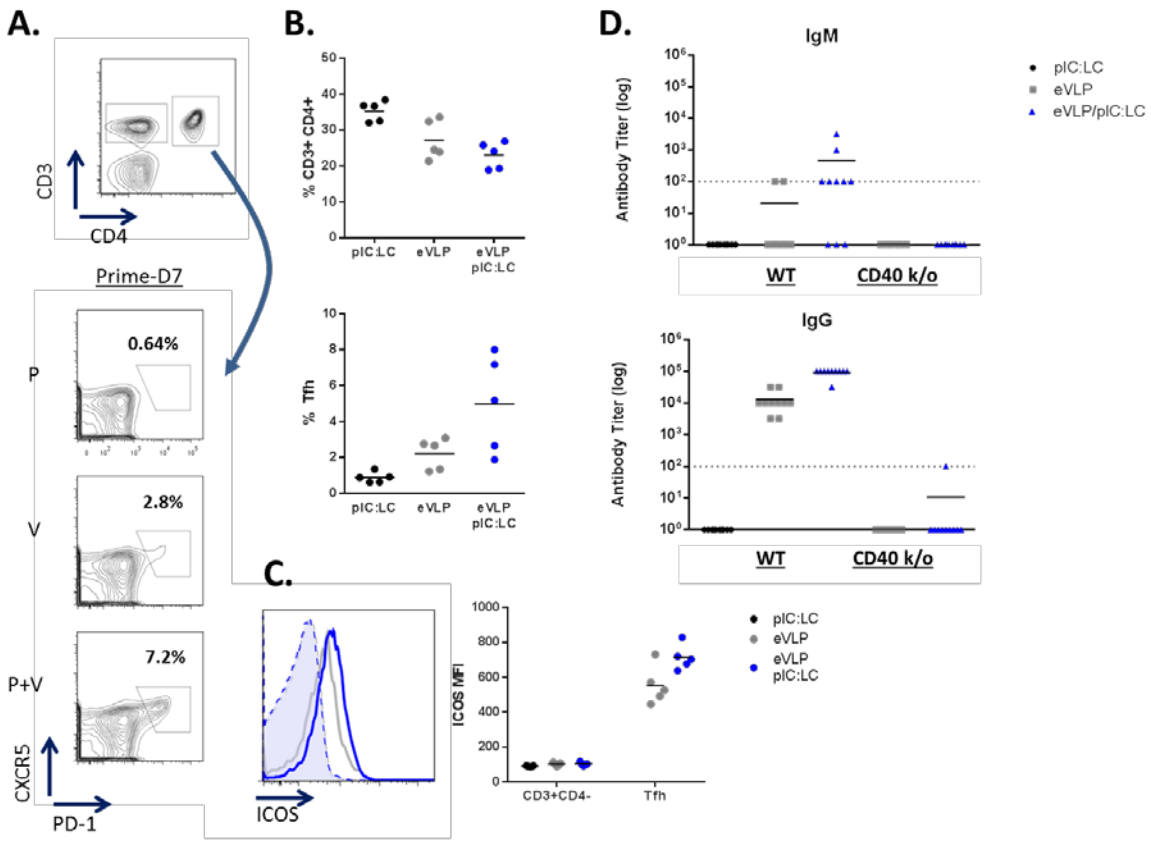

Figure 3. VLP-mediated humoral responses are T cell-dependent. C57BL/6 mice were vaccinated with 10 ug (GP content) of VLP or 10 ug of VLP with 10 ug of Hiltonol. Single cell suspensions from the DLN were then stained with CD4, CD3, CXCR5, PD-1, ICOS and live/dead dye and collected on a BD FACS Canto II. A) Representative gating of D7 prime Tfh subsets. B) top, relative percentage of CD3+CD4+ and bottom, Tfh frequency. C) left, representative ICOS surface expression of CD3+CD4- (shaded) and CD3+CD4+PD1+CXCR5hi (open) Tfh cells; right, mean fluorescent intensity of respective T cell subsets and vaccination. (mean, s.e.m; n=5) D) C57BL/6 mice or CD40 knockout mice were vaccinated with 10 ug (GP content) of VLP with and without Hiltonol at Day 0 and Day 21. Serum was collected at D35 (D14 post-boost) and EBOV-GP specific IgG and Ig M responses were measured by ELISA. Titers were calculated by reciprocal end-point dilutions with background set at control absorbance + 0.2 O.D. (n=10/group). Dash lined represents level of detection.

EBOV protection in the absence of humoral responses

Previous studies using B cell deficient animals (μMT, Jh-/-) and anti-CD20 B cell depleting studies have suggested that vaccine-induced antibody responses are obligate for EBOV protection. We therefore tested the protective contribution of humoral immunity using CD40 -/- animals which displayed a complete lack of EBOV-specific antibody responses. As previously demonstrated, prime-boost

vaccination of WT animals with eVLP or eVLP/Hiltonol resulted in acute EBOV protection against a lethal challenge of ma-EBOV (Fig. 4a). Surprisingly, we also observed partial protection in eVLP vaccinated CD40 -/- animals with no significant effect observed with inclusion of adjuvant (Fig. 4a). While control animals succumbed to EBOV infection by D10, in two separate challenge studies, we observed a combined 60% (n=24/40) protection in eVLP vaccinated animals without detectable antibody responses. These unexpected results led us to speculate that despite the lack of TI responses with eVLP vaccination, live-viral EBOV infection may result in the generation of protective low-affinity antibody responses. To test this hypothesis, we analyzed antibody responses following EBOV infection in the animals that survived lethal challenge. As seen following eVLP vaccination, all surviving WT animals had robust IgG titers against EBOV GP; however, CD40 deficient animals displayed a near complete absence of antibody responses even following live-viral EBOV infection (Fig. 4b). Of the 14 CD40 -/- surviving animals analyzed after lethal challenge, only 3 (3/14) displayed marginal IgG titers and none (0/14) established IgM responses (Fig. 4b). We next tested if sterile immunity was generated within surviving animals. Following Day 28 post primary EBOV infection, animals were back challenged with a lethal dose of ma-EBOV. Consistent with initial survival results, animals were 100% protected against subsequent EBOV infection including those without detectable antibody responses (Fig. 4c). Together, this supports that T cell responses are required to generate EBOV-specific antibody responses against both eVLP vaccination and live-viral infection. Further, we discovered an unexpected result that protection against EBOV can be achieved in the absence of humoral immunity.

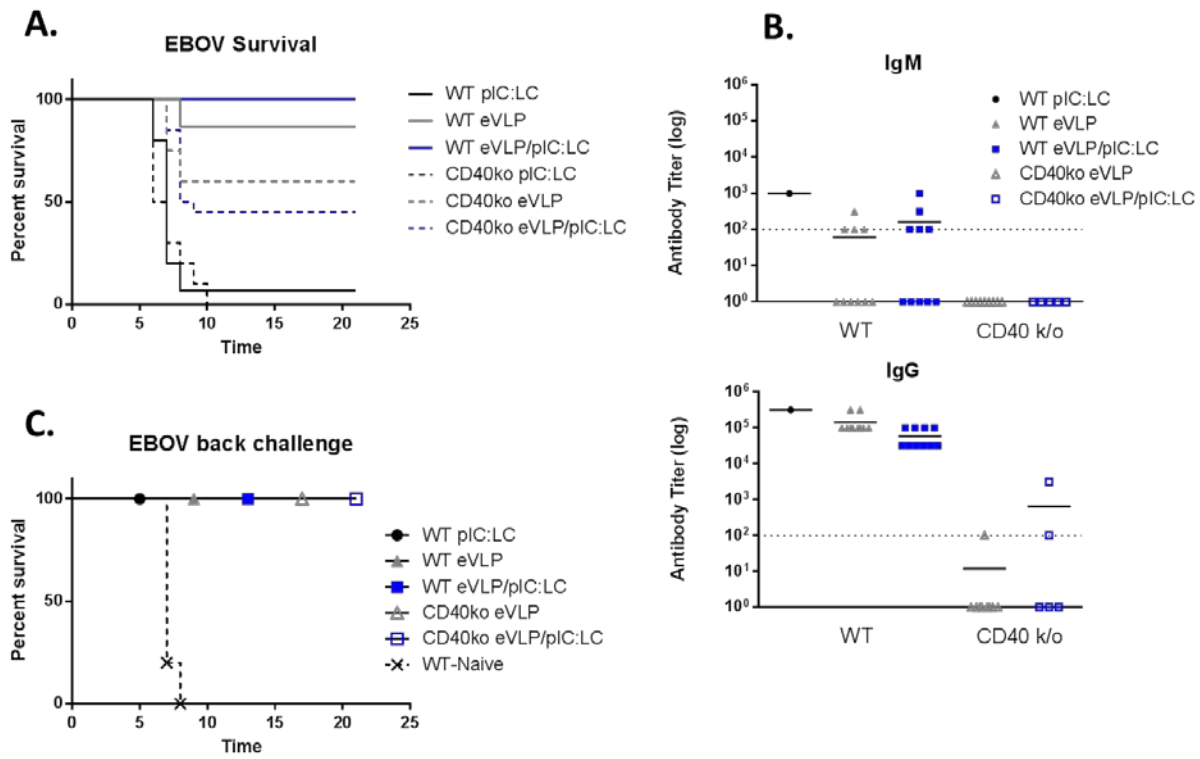

Figure 4. EBOV survival without humoral immunity. A) C57BL/6 mice or CD40 knockout mice were vaccinated with 10 ug (GP content) of VLP with and without Hiltonol at Day 0 and Day 21. On D49 (4-weeks post vaccination) animals were challenged with a lethal dose of ma-EBOV. Combined survival from 2 independent vaccination studies (n=15/group for Wt animals; n=20/group for CD40 ko vaccinated; n=10/group CD40 ko control) B) Serum was collected from surviving animals on Day 21 post-EBOV infection and GP-specific IgG and Ig M responses were measured by ELISA. Titers were calculated by reciprocal end-point dilutions with background set at control absorbance + 0.2 O.D. Dash lined represents level of detection. C) Animals surviving initial EBOV infection was back challenged with a lethal dose of ma-EBOV on D28 post initial infection and monitored for protection.

Antibody-independent but B cell-dependent mechanisms promote EBOV protection

The finding of EBOV survival in the complete absence of antibodies is contradictory to previous reports. We therefore wished to further define the *in-vivo* requirement for antibodies in vaccine protection. Aicda (AID) mediates both somatic hypermutation and class-switching of GC B cells within the follicle of TD immune responses. In the absence of AID, B cells fail to generate high-affinity IgG isotype antibodies; however, functional B cells are still generated. Using AID deficient (AID -/-) animals we further tested the role of humoral immunity in EBOV protection. WT or AID -/- animals were prime-boost vaccinated with

198 eVLP as previously described. Consistent with eVLP vaccinations, WT mice generated robust IgG
199 responses; however, AID -/- animals had a complete absence of class-switched EBOV-GP specific IgG
200 titers (Fig. 5a). To determine the protective role of these IgG responses, WT and AID -/- animals were
201 challenged with a lethal dose of ma-EBOV. In accordance with our CD40 -/- studies, we observed ~60%
202 protection against EBOV infection in animals lacking IgG antibody responses (Fig. 5b). Interestingly, eVLP
203 vaccinated AID -/-animals mounted marginal IgM responses not seen in WT animals; however, this does
204 not appear to be required for protection (Fig. 5a). Collectively, these studies support that while EBOV
205 antibody responses promoted protection, survival in the absence of humoral immunity can be achieved.

206 Previously defined humoral roles for several EBOV vaccine platforms, including those in FDA clinical
207 trials, have suggested an absolute requirement for antibodies in EBOV protection. However, these
208 studies have traditionally been conducted in animals which display a developmental block in B cell
209 lymphopoiesis and therefore have a complete loss of the B cell compartment. To further characterize
210 the role of B cells in EBOV vaccine responses, we revisited the IgM transmembrane domain deleted
211 (μMT) mouse model which has been previously used to describe EBOV antibody roles. As above, WT
212 and μMT mice were prime-boost vaccinated with eVLP and then challenged with a lethal dose of ma-
213 EBOV 4 weeks post vaccination. While control animals succumbed to EBOV infection by D7 post
214 infection, WT eVLP vaccinated mice were fully protected (Fig. 5d). Consistent with previous reports,
215 eVLP vaccinated μMT mice failed to establish EBOV-GP specific antibody responses and were not
216 protected against EBOV infection (Fig. 5c, d). Interesting, vaccination failed to protect in the absence of
217 B cells but EBOV survival without antibodies was achievable, potentially suggesting additional antibody-
218 independent roles for B cells in promoting vaccine-mediated protection.

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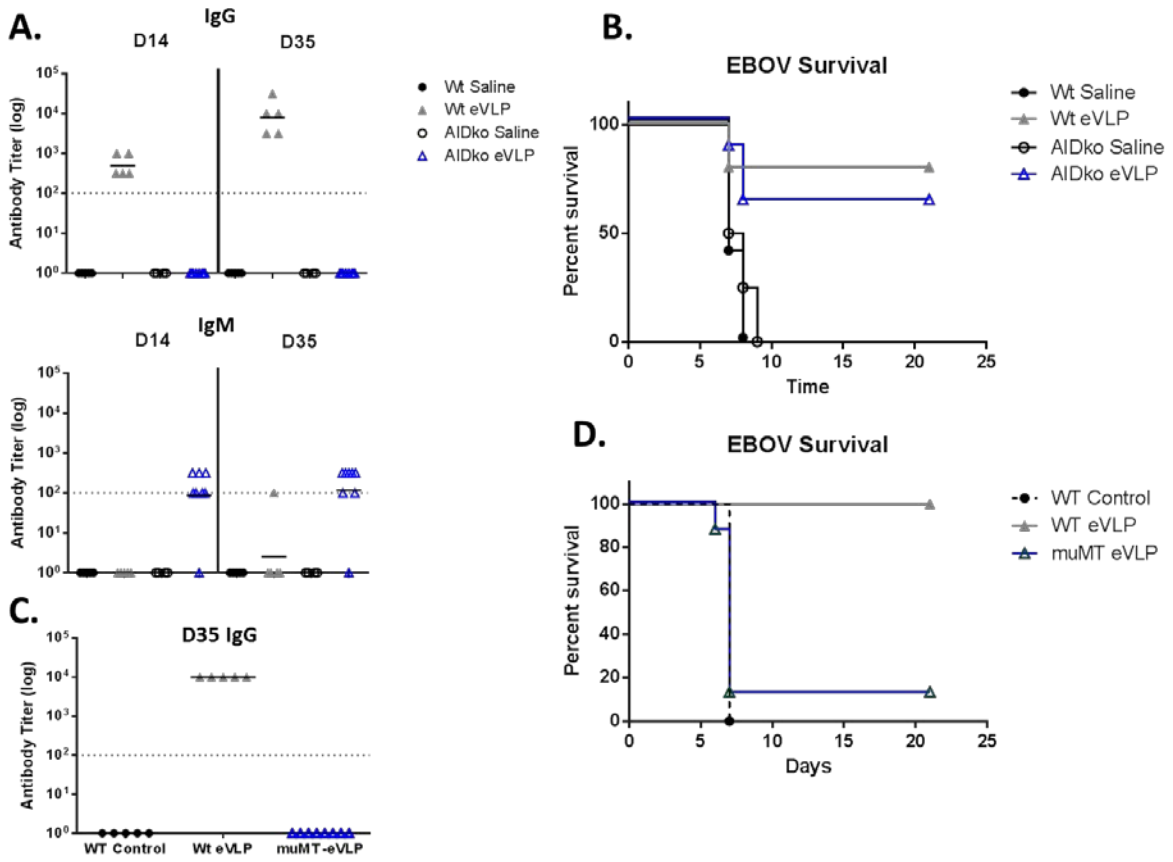

Figure 5. Antibody-independent B cell responses promote EBOV protection. A) C57BL/6 mice or AID knockout mice were vaccinated with 10 ug (GP content) on Day 0 and Day 21. Serum was collected on D14 and D35 (D14 post-boost) and EBOV-GP specific IgG and Ig M responses were measured by ELISA. Titers were calculated by reciprocal end-point dilutions with background set at control absorbance + 0.2 O.D. Dash lined represents level of detection. B) Animals were challenged with a lethal dose of ma-EBOV on D49 (n=5 WT mice/group; n=8 AID ko vaccinated, n=4 AID saline). C) C57BL/6 mice or uMT knockout mice were vaccinated and serum was collected as above. D) Survival of animals challenged with a lethal dose of ma-EBOV on D49 (n=5 WT mice; n=8 uMT ko).

Together, our findings offer a greater understanding of how *in-vivo* Ebola humoral immune responses are acquired during vaccination or infection. We define that Ebola GP humoral responses generated against both eVLP and live-virus infection are established through T cell-dependent mechanisms. In addition, we provide evidence that the clinical grade adjuvant, Hiltonol, augments protective humoral immune responses through induction of GC B cell and T follicular help interactions. Unexpectedly and contrary to previous works, we demonstrate that while antibody responses can promote EBOV

protection, survival in the absence of antibodies can be achieved. This finding may be of particular clinical relevance given the lack of a definitive EBOV immune correlate despite on-going FDA vaccine trials and the likely requirement of vaccine licensure through the "animal rule" to demonstrate efficacy. Furthermore, our data suggests that individuals with primary and secondary immunodeficiency affecting productive antibody responses (i.e. Hyper-IgM syndrome, BAFF/BAFFR disorders, common variable immunodeficiency) could acquire EBOV protection.

References (working)

Figure legends

Figure 1. Characterization of short-term and memory EBOV GP antibody responses following eVLP vaccination. C57BL/6 mice were vaccinated with 10 ug (GP content) of VLP or 10 ug of VLP with 10 ug of Hiltonol at Day 0, D21 and D>5 months. Serum was collected at the indicated time-points and EBOV-GP specific IgM and IgG responses were measured by ELISA. A,B) EBOV-GP specific IgG titer (A) and viral neutralization (B) during acute phase protection. C) Durable IgM and IgG antibody responses. Titers were calculated by reciprocal end-point dilutions with background set at control absorbance + 0.2 O.D. (n=3). Arrows indicate vaccination time points.

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Material and Methods

Reagents.

PolyICLC (Hiltonol) was provided by Oncovir, Inc. VLP were manufactured by as previously described. Briefly, 293T were transfected with Ebola Zaire (kikwit) virus glycoprotein and VP40 and supernatants were collect at 72hrs post-transfection. VLP were then purified by sucrose gradient and GP content determined by western. To ensure sterility, VLP were irradiated at 1e6 rad and contained less than 25 EU/ml endotoxin and less than 10 colony forming units (CFU) of bacteria per vaccination. VLP were maintained at -80 degree Celsius and diluted in sterile saline and/or combined with polyICLC prior vaccination.

Mouse Strain and vaccinations

C57BL/6 mice (NCI Charles River, Jackson), CD40 deficient (Jackson), uMT (Jackson) and AID deficient (Kind gifts from Pat Gearhart, NIAIA, Baltimore and Raffaello Casselas, NIH, Bestheda) mice were i.m.

vaccinated with eVLP (10 ug GP content) or eVLP (10ug GP content) with Hiltonol (10 ug, Oncovir) at 3 week intervals (Day0, Day21).

EBOV GP Serum ELISA

Blood was collected at the indicated time points in SST tubes. ELISA plates were coated at 4 degree overnight with recombinant mammalian Ebola GP at 2 ug/ml in PBS. Plates were washed 3X with PBS-T (PBS, 0.1% Tween-20) and then blocked 2 hours at RT with PBS-T 5% non-fat milk. Serum was diluted by half-log dilutions starting at 1:100 and incubated for 1 hr on GP-coated plates. Plates were then washed 3X with PBS-T, incubated with the indicated secondary HRP-antibody for 45 minutes, and then washed 3X with PBS-T. ELISA was developed using TMB substrate/stop solution and measured on a Tecan plate reader. Absorbance cut-off was determined as background + 0.2 O.D.

Flow cytometry

Single cell suspensions of draining lymph nodes and spleen was collected at the indicate time points. Cells were washed using FACS buffer (PBS, 0.5% BSA and 2mM EDTA), RBC lysed and then subsequently counter-stained with the following B cell antibodies: B220, IgM, IgD, IgG1, CD38, CD138, and GL-7 (all purchased from BD). All samples were Fc blocked (anti-CD16/CD32, BD) and viability (live/dead aqua; Invitrogen) stained prior to antibody staining. Data was collected on BD FACS Canto II or BD Aria II and analyzed using Flowjo (Treestar).

Neutralization assay

Neutralizing antibody titers from serum samples were determined using rVSV particles expressing the Zaire GP and GFP (ref). rVSV-EBOV-GP-eGFP (kind gift of XXX) particles were propagated as previously described. Briefly, VERO E6 cells inoculated with rVSV-EBOV GP-eGFP were maintained until greater than 90% c.p.e. Supernatants were cleared of cellular debris by 0.45 μm filtering and centrifugation at

1,000 RCF for 10 minutes. Virions were then concentrated (4^{o}C for 2hrs at 27,900 RPM), titer determined and aliquoted. For neutralization assays, serum dilutions beginning at 1:10 were incubated at 37^{o}C for 1hr with rVSV and 5% v/v guinea pig complement (Cedarlane Hemo-lo). rVSV/serum complexes were then incubated with 1.0 * 10^5 293F cells at 1.0*10^6 cells/mL for 18-20 hrs at 37^{o}C. Infection percentage, as determined rVSV-GFP expression, was determined using FACS. Data was collected on BD Canto II and LSR II. Neutralization was calculated by normalizing infection percentages to infections carried out in the presence of control serum from non-vaccinated animals.

EBOV Challenge

Mice were inoculated with a target titer of 1,000 pfu of ma-EBOV (need to include actual ID number) on Day 28 post last vaccination. All studies were conducted in the USAMRIID Biosafety Level 4 containment facility. Beginning on Day 0 and continuing for the duration of the in-life phase, clinical observations were recorded and animals were closely monitored for disease progression. Moribund animals were euthanized based on Institutional approved clinical scoring.

Disclaimers

Army:

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

IACUC:

Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment

345 and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide
346 for the Care and Use of Laboratory Animals, National Research Council, 2011.

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